TRANSGENIC PATHOGEN-RESISTANT ORGANISM

This is a divisional of application No. 08/457,797, filed on Jun. 1, 1995, now U.S. Pat. No. 5,689,045, which is a continuation of Ser. No. 08/134,416, filed on Oct. 8, 1993, now abandoned.

FIELD OF THE INVENTION

The invention relates to a pathogen-resistant organism and to a process for generating it.

BACKGROUND OF THE INVENTION

It is known in the state of the art that infestations of a plant by pathogens causes a series of different reactions. These include, for example, changes in the cell wall structure, the synthesis of phytoalexins which have antimicrobial activity, the accumulation of so-called PR proteins (pathogenesis-related), protease inhibitors and enzymes with hydrolytic functions (Hahlbrock and Grisebach in Ann. Rev. Plant. Physiol., 30 (1979), 105–130).

Many pathogens (fungi and insects) have chitin as a constituent of their cell wall. By contrast, plants possess no chitin. It has now been demonstrated in some cases that there is enhanced production of chitinases in plants after infestation by pathogens. Chitinases are among the enzymes with hydrolytic functions and they catalyze chitin breakdown. It has now been possible to show that plants acquire an increased resistance to pathogens by the production of chitinases.

It is furthermore known to use a gene from barley plants whose gene product codes for an inhibitor of fungal protein synthesis. The incorporation of a corresponding inhibitor gene in transgenic plants led to improved resistance to fungi.

Finally, it has also been disclosed that the use of a polypeptide from *Aspergillus giganteus* is able to protect, by virtue of its antifungal activity, plants from infestation by fungi.

However, given this state of the art there is a need to provide further transgenic pathogen-resistant organisms. Moreover, the organisms which are particularly desired are those whose resistance is increased overall by comparison with the known organisms or is extended with respect to the number of possible pathogens.

This problem is solved by a transgenic pathogen-resistant organism having the features of the present invention.

The invention is based on the surprising finding that the incorporation of at least two different genes with pathogen-inhibiting action into the genome of an organism assists the latter to resist pathogens to an extent going far beyond an additive effect of each of the genes on its own.

The dependent claims indicate further embodiments of the invention.

The genes can code for gene products which reduce the vitality of fungi. In particular, the genes can be of fungal, bacterial and plant, animal or viral origin. In particular, the gene products have properties which promote resistance to fungi. The gene products are chitinase (ChiS, ChiG), glucanase (GluG), protein synthesis inhibitor (PSI) and antifungal protein (AFP).

The transgenic pathogen-resistant organism can be a plant, and tobacco, potato, strawberry, corn, rape or tomato plants are preferred.

The invention also relates to DNA-transfer vectors with inserted DNA sequences as are indicated in detail in this description.

The invention furthermore relates to a process for the generation of pathogen-resistant organisms as are described herein, wherein at least 1 gene with pathogen-inhibiting action is transferred into the genome of an organism, and the pathogen-resistant organism is obtained

(a) by crossing the organism with another, optionally transgenic, organism which contains at least one other gene with pathogen-inhibiting action, and subsequently selecting, and/or

(b) by transformation of this other gene with pathogen-inhibiting action into the organism. The process can be used with DNA-transfer vectors with inserted DNA sequences corresponding to a gene with pathogen-inhibiting action as described herein.

Finally, the invention relates to a process for the generation of pathogen-resistant organisms, wherein vectors which comprise more than one gene with pathogen-inhibiting action are used for the transformation into the genome of an organism.

The invention also relates to a process for ensuring the resistance of organisms to pathogens, characterized in that the organism used is a transgenic pathogen-resistant organism according to the present invention or an organism whose genome contains at least one gene complying with the definitions used herein, and at least one substance which is not expressed by the organism but corresponds to any other one of the gene products complying with the definitions given in this application is applied to the organism.

It was possible to achieve the synergistic effects very particularly with transgenic pathogen-resistant organisms to which the gene sequences which coded for proteins of the attached sequence listings A to E, or corresponded to the latter, were transferred or transfected.

ChiS:

A DNA fragment which is 1.8 Kb in size, that codes for a chitinase called ChiS (SEQ ID NO: 8) was isolated from the soil bacterium *Serratia marcescens*. In vitro investigations with purified ChiS protein showed that it is able effectively to inhibit the growth of fungi, even in low concentrations. The reason for the inhibition is that the ChiS protein has a chitinase activity which is able to damage the tips of the fungal hyphae. In this way the fungus is unable to grow further and is inhibited.

PSI:

The PSI gene originates from barley and codes for a protein which inhibits protein synthesis by fungi. In vitro tests show that even low concentrations of PSI are sufficient to inhibit various fungi such as, for example, *Rhizoctonia solani*.

AFP:

It is possible for a polypeptide which has antifungal activity to be isolated from the fermentation broth of *Aspergillus giganteus* and to be sequenced. This polypeptide is suitable as antifungal agent, for example as spraying agent and as preservative for industrial products and human and animal foods. It can furthermore be combined with other substances which have pesticidal activity, fertilizers or growth regulators. Inhibitory activities against fungi were detectable inter alia against various Aspergillus, Fusaria, Phytophthora and Trichophyton species.

ChiG and GluG:

Two genes which code, respectively, for a chitinase (ChiG) and glucanase (GluG) can be isolated from certain types of barley. Purified ChiG protein or GluG protein inhibits various phytopathogenic fungi in vitro (inter alia

Rhizoctonia solani) (see R. Leah et al., Journal of Biological Chemistry, Vol. 266, No. 3 (1991), pages 1564–1573).

SUMMARY OF THE INVENTION

The inventors have now found, completely surprisingly, that an at least binary combination of expression of PSI, AFP, ChiS, ChiG or GluG leads to synergistic effects in respect of the acquired resistance to fungi in transgenic plants. In particular, the effects of the individual substances in the combination are markedly exceeded. These include resistance to the fungus *Rhizoctonia solani,* Sclerotinia infestation, Botrytis infestation, etc.

Combinations according to the invention are (DNA and/or polypeptides):

(binary combinations)

ChiS, GluG; ChiS, PSI; ChiS, ChiG; ChiS, AFP; GluG, PSI; GluG, ChiG; GluG, AFP; PSI; ChiG; PSI, AFP; (ternary combinations)

ChiS, GluG, PSI; ChiS, GluG, ChiG; ChiS, GluG, AFP; GluG, PSI, ChiG; GluG, PSI, AFP; PSI, ChiG, AFP; ChiG, AFP, GluG

(quaternary combinations)

ChiS, GluG, PSI, AFP; ChiS, GluG, PSI, ChiG; (quinary combination)

ChiS, GluG, PSI, AFP, ChiG

The invention furthermore relates to the combined use of the proteins with pathogen-inhibiting action, preferably ChiS, PSI, AFP, ChiG and GluG, against pathogens. Combined use also means in this context that at least a first pathogen-inhibiting substance is expressed by the organism and at least a second substance which has pathogen-inhibiting action is applied to the organism from outside.

The agents according to the invention also include those which contain the abovementioned proteins in at least binary combination. The agents according to the invention can contain other active substances besides the proteins. The other active substances can be pesticides, fertilizers and/or growth regulators, and the agents according to the invention can be prepared in various formulations such as concentrates, emulsions, powders, formulations or carriers, mixtures with other active substances, etc. The ChiS/PSI and AFP/PSI combination is particularly preferred. These proteins can be used particularly effectively to inhibit the growth of *Rhizoctonia solani,* especially in tobacco crops.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the effects of AFP and PSI on *Rhizoctonia solani.*

FIG. 2 shows the effects of ChiS and PSI on *Rhizoctonia solani.*

DETAILED DESCRIPTION OF THE INVENTION

The invention also relates to the use in a process according to the invention of a DNA sequence which codes at least for a polypeptide of sequences A to E, in which sequence A is the sequence of a 60 amino acid AFP protein (SEQ ID NO: 2); sequence A' is the sequence of 51 amino acid AFP protein (SEQ ID NO: 3); sequence B is the sequence of the PSI protein (SEQ ID NO: 5); sequence B' is the sequence of a protein encoded by an incomplete PSI-cDNA clone (SEQ ID NO: 7); sequence D is the sequence of the ChiG protein (SEQ ID NO: 10); and sequence E is the sequence of the GluG protein (SEQ ID NO: 12) or to a pathogen-resistant organism, where its genome contains at least two different genes under the control of active promoters with pathogen-inhibiting action, where the genes are in each case selected from the group of sequences A to E, in which sequence A is the sequence of a nucleic acid (SEQ ID NO: 1) which comprises a region encoding AFP protein; sequence B is the sequence of a nucleic acid (SEQ ID NO: 4) which comprises a region encoding PSI protein; sequence B' is the sequence of a nucleic acid (SEQ ID NO: 6) which was identified as a portion of an incomplete PSI-cDNA clone; sequence C is the sequence of a nucleic acid (SEQ ID NO: 8) encoding ChiS protein; sequence D is the sequence of a nucleic acid (SEQ ID NO: 9) which comprises a region encoding ChiG protein; and sequence E is the sequence of a nucleic acid (SEQ ID NO: 11) which comprises a region encoding GluG protein. The invention furthermore includes DNA sequences which hybridize with a DNA sequence which codes for polypeptides of amino-acid sequences A to E, in which sequence A is the sequence of a 60 amino acid AFP protein (SEQ ID NO: 2); sequence A' is the sequence of a 51 amino acid AFP protein (SEQ ID NO: 3); sequence B is the sequence of the PSI protein (SEQ ID NO: 5); sequence B' is the sequence of a protein encoded by an incomplete PSI-cDNA clone (SEQ ID NO: 7); sequence D is the sequence of the ChiG protein (SEQ ID NO: 10); and sequence E is the sequence of the GluG protein (SEQ ID NO: 12), where these DNA sequences can be of natural, synthetic or semisynthetic origin and can be related to the abovementioned DNA sequence by mutations, nucleotide substitutions, nucleotide deletions, nucleotide insertions and inversions of nucleotide sequences, and for a polypeptide with pathogenic activity. The invention furthermore relates to a recombinant DNA molecule which contains at least one DNA sequence which accords with the preceding statements, where this DNA molecule can be in the form of a cloning or expression vector.

The invention relates to appropriate host organisms and intermediate hosts which are transformed with a recombinant DNA molecule which accords with the preceding statements. Preferred as intermediate host in the generation of a pathogen-resistant transgenic organism are strains of bacteria, in particular so-called Agrobacteria strains.

The invention furthermore relates to the transgenic pathogen-resistant organisms obtained by the process according to the invention, in particular tobacco, potato, corn, pea, rape and tomato plants.

The DNA sequences according to the invention are, as a rule, transferred together with a promoter. Promoter sequences are recognized by the plant transcription apparatus and thus lead to constitutive expression of the gene associated with them in plants. The promoter can, however, also be pathogen-inducible and/or wound-inducible (WUN1) and/or tissue-specific and/or development-specific.

The genetic manipulation operations necessary for carrying out the invention, especially for expression of the gene in plants, are generally known. See for example the publication by Maniatis et al. in "Molecular cloning: A laboratory manual", Cold Spring Harbor (1982).

The invention is explained in detail in the following examples.

All the standard methods of molecular biology were carried out, unless otherwise indicated, as described by Maniatis et al. "Molecular cloning: a laboratory manual", Cold Spring Harbor (1982).

The DNA (SEQ ID NO: 1; SEQ ID NO: 4; SEQ ID NO: 6; SEQ ID NO: 8; SEQ ID NO: 9; SEQ ID NO: 11) coding

for amino-acid sequences A to E (SEQ ID NO: 2; SEQ ID NO: 3; SEQ ID NO: 5; SEQ ID NO: 7; SEQ ID NO: 10; SEQ ID NO: 12) was initially cloned in a manner known per se and then transferred by conjugation into *A. Tumefaciens* LBA 4404 (A. Hoekema et al., Nature 303, 179–180). This took place by the method described by Van Haute et al. in EMBO J. 2, 411–418 (1983).

The transfer of DNA into that Agrobacterium was checked by isolating Agrobacterium DNA by the method described by Ebert et al. in Proc. Natl. Acad. Sci. USA 84 5745–5749 (1987). Restriction cleavage of the DNA, transfer to Hybond-N membrane (Amersham) and hybridization with a radioactively labeled DNA probe provided information about successful DNA transfer into the Agrobacterium.

The transformed Agrobacterium was then used to transform tobacco, rape, strawberry, tomato and potato plants.

The LBA4404 Agrobacteria required for the infection were initially cultivated in selective antibiotic medium (P. Zambrisky et al. in EMBO J., 1, 147–152 (1983)), sedimented by centrifugation and washed in YEB medium without antibiotics (YEB=0.5% meat extract; 0.2% yeast extract; 0.5% peptone; 0.5% sucrose; 2 mM $MgSO_4$). After renewed sedimentation and taking up in $MgSO_4$ it was possible to use the bacteria for the infection.

The so-called leaf disk method was used for the infection.

Sterile leaves were used for the leaf disk infection. Leaf pieces about 1 cm in size are dipped in the previously described Agrobacteria suspension and subsequently transferred to 3 MS medium (medium described by T. Murashige and F. Skoog in Physiol. Plant., 15, 473–497 (1962); 3MS=MS–3% sucrose). After incubation at 25° C. to 27° C. with 16 hours of light for two days, the leaf pieces were transferred to MSC16 medium (according to T. Murashige (see above); MSC16=MS+0.5 μg/ml BAP+0.1 μg/ml NAA+100 μg/ml kanamycin sulfate+500 μg/ml Claforan). Shoots appearing after 4–6 weeks were cut off and transplanted to MSC15 medium (according to Murashige (see above); MSC15=MS+2% sucrose, 500 μg/ml Claforan+100 μg/ml kanamycin sulfate). Shoots with root formation were analyzed further.

Monocotyledonous plants (including corn), but some dicotyledonous plants too, were transformed by direct gene transfer into protoplasts. These protoplasts were subsequently regenerated to intact plants (Example: J. Potrykus in Biotechnology 8 (1990), 535).

The resulting transgenic plants were infected with the fungus *Rhizoctonia solani* for testing purposes. For this purpose, fungal cultures were grown and thoroughly mixed in standard soil. This soil was then distributed in a dish and planted with the plants to be tested.

For the evaluation, each plant on a dish was assigned a value from 0 to 3. It was possible to calculate from this for each plant line an index which resulted from the sum of the values. The classification is as follows:

0=no symptoms (healthy)

1=slightly reduced size (compared with a non-infected control); no or very slight visible infestation

2=severe reduction in growth; severe symptoms of infestation

3=dead

The rating is carried out in each case 14 days after the start of the series of tests.

EXAMPLE 1:

Fungus inhibition test with combined proteins

The intention initially was to show that the proteins used here have synergistic effects in their combination. Fungal growth tests in vitro were carried out for this purpose.

These entailed a defined amount of *Rhizoctonia solani* fungal mycelium being mixed with 100 μl of potato dextrose solution and incubated in microtiter plates at 25° C. In this test there is a linear correlation between the growth of the fungus and the increase in the optical density at 405 nanometers. The inhibitory effect of proteins can be detected from a smaller increase in the optical density.

2–3 mycelium balls were taken from a liquid culture of *R. solani,* mixed with 100 μl of KGB medium in an Eppendorf vessel and carefully homogenized with a glass mortar. This suspension was then mixed with 10 ml of KGB medium and passed through a sterile 100 μm screen. The optical density of this mycelium fragment suspension (100 μl aliquot) was adjusted to a value of 0.06–0.07 at 405 nanometers by adding medium. 100 μl samples were placed on a microtiter plate and mixed with the proteins to be tested. 7 parallels were measured per mixture. Mixtures which were mixed with the corresponding amounts of buffer served as controls. The plates were incubated in the dark at 25° C. for 48 hours, and the optical density of the cultures was measured at regular intervals.

Calculation of whether two proteins act together in an additive synergistic or antagonistic manner in the inhibition of fungal growth is possible from the measured data with the aid of the Colby formula which is described hereinafter and generally used (S. R. Colby in Wheeds, 15 (1967), 20–22).

To do this it was initially necessary to calculate the growth inhibition E to be expected theoretically with an additive behavior (the expected efficacy). This is given by:

$$E=W1+W2-((W1\times W2)/100)$$

where W1 and W2 indicate the efficacies of the individual proteins, which is defined as that percentage deviation of the growth plot (in the presence of the protein) from the untreated control. The efficacy for a protein (at a defined time in the growth plot) is given by:

$$W1=(OD(K)-OD(P))/OD(K)\times 100 \text{ (percent)}$$

In this, OD(K) is the optical density of the untreated control and OD(P) is the optical density of the culture treated with the protein.

Thus, on combined use of two proteins, the following statements were possible: if the efficacy G measured in the experiment is identical to the expected value E, the behavior is additive. If, on the other hand, G is greater than E, the behavior is synergistic.

Using this test model, it emerged that the proteins ChiS, PSI, AFP, ChiG and GluG used in the Example surprisingly have synergistic inhibitory effects on various fungi, and these effects were achieved both by the combination of two types of protein and by multiple combination of the above-mentioned proteins.

For example, the following values were determined from the combination of ChiS and PSI protein and from the combination of AFP protein and PSI protein on the fungus *Rhizoctonia solani* (in each case two different ChiS and AFP concentrations with a constant RIP concentration):

ChiS+PSI:

The expected values were: E1=29.9% and E2=44.5%

The measured values were: G1=60.4% and G2=64.1%

The proteins ChiS and PSI therefore act together in a synergistic manner in the inhibition of the growth of *R. solani*.

FIG. 1 shows the results obtained with the combination of the proteins and with the individual substances. According to the Figure, various ChiS concentrations (0.5 μg/ml and 0.05 μg/ml) are combined with PSi protein (1.0 μg/ml.

AFP+PSI:

The expected values were: E1=39.9% and E2=41.9%

The measured values were: G1=57.7% and G2=65.4%

The AFP and PSI combination also according to this shows a synergistic inhibition of growth of the fungus *R. solani*. FIG. 2 indicates the test results with various AFP concentrations (0.4 μg/ml and 0.04 μg/ml) combined with PSI protein (1.0 μg/ml).

EXAMPLE 2:

Transgenic plants

In order to obtain the organisms according to the invention with DNA sequences which act together synergistically, initially transgenic plants which contained at least one of the genes which act together synergistically were generated.

ChiS in transgenic slants

Initially a ChiS gene was fused to plant regulatory sequences.

A ChiS gene 1.8 Kb in size was sequenced by using synthetic oligonucleotides in the dideoxy sequencing method of Sanger et al. in Proc. Natl. Acad. Sci. USA, 74 (1977), 5463–5467.

The 35S promoter originating from cauliflower mosaic virus (CamV) (400 bp (according to Töpfer et al. in Nucl. Acid. Res., 15 (1987), 5890)) underwent transcriptional fusion to the ChiS gene. The termination signal, which is 0.2 Kb in size, of the 35S gene of CamV, whose functionality in dicotyledonous plants is known, was used 3' from the ChiS gene. The chimeric gene 35S-ChiS was cloned into the pLS034 vector by means of the *Agrobacterium tumefaciens* transformation system in tobacco and potato plants, and kanamycin-resistant plants were regenerated.

It was possible to detect both the ChiS gene and the corresponding mRNA as well as the gene product protein in the resulting plants.

PSI in transgenic plants

PolyA RNA was initially isolated from ripe barley seeds (Hordeum vulgare L. cv. Piggy) and deposited in a cDNA gene bank λ-gt-11-phages. The details of the process are to be found in R. Lea in Plant. Biol., 12 (1989), 673–682. Monospecific PSI antibodies were then used to identify CDNA clones.

Subsequently, the PSI-positive λ-gt-11-phages were isolated, cloned further and sequenced by the dideoxy sequencing method of Sanger et al. indicated above. The DNA cloned into *E. coli* was then transferred in the manner described above by conjugation into Agrobacterium LBA4404.

Both the transferred gene and mRNA and gene product were detectable in corresponding transgenic tobacco, potato, rape, strawberry and tomato plants.

AFP in transgenic plants

For the cloning in the vector, the cDNA sequence of the antifungal peptide is provided with ends which can be ligated into BamH1 and Sal1 restriction cleavage sites. The cloning vector used was pDH51 (Pietrzak et al. in Nucl. Acids Res. 14 (1986), 5857). The vector pDH51 was opened with the restriction enzymes BamH1 and Sal1 between promoter and terminator. The vector pDH51 is a pUC18 derivative which contains promoter and terminator sequences of the 35S transcript from cauliflower mosaic virus. These sequences are recognized by the plant's transcription apparatus and lead to strong constitutive expression of the gene associated with them in plants. The DNA of the antifungal peptide is then cloned via the BamH1 and Sal1 cleavage site into the vector. Finally, the transcription unit—promoter, gene and terminator—is cut out of the vector using the restriction enzyme EcoRI and cloned into a plant transformation vector. The following vectors and their derivatives can, for example, be used as plant transformation vector:

pOCA18 (Olszewski et al. in Nucl. Acids Res., 16 (1988), 10765) pPCV310 (Koncz and Shell in MGG 204 (1986), 383) and pBin19 (Bevan et al. Nucl. Acids. Res. 12 (1984), 8711)

After the transcription unit and the vector had been ligated via the EcoRI cleavage site, the construct was conjugated into the Agrobacterium strain MP90RK (Koncz and Shell (see above)) or IHA101 (Hood et al. in J. Bacteriol. 168 (1986), 1291).

Transgenic tobacco, potato, strawberry, rape and tomato plants were then transformed by the method described above. Transformed shoots are selected on the basis of the cotransferred resistance to the antibiotic kanamycin. Expression of the antifungal protein in the transformed crop plants was checked and confirmed by DNA analysis (Southern blotting), RNA analysis (Northern blotting) and protein analysis with specific antibodies (Western blotting).

ChiG and GluG in transgenic plants

ChiG- and GluG-transgenic plants which were both Southern-, Northern- and Western-positive were obtainable in analogy to the plants described above.

ChiS, PSI, AFP, ChiG, GluG in transgenic monocotyledonous plants

It was possible by means of direct gene transfer to integrate the abovementioned genes into the genome of monocotyledonous plants such as, for example, corn. This resulted in transgenic plants which were Southern- and Northern- and Western-positive.

Combination of various fungus-resistance genes in transgenic plants

The previously obtained tobacco, corn, rape, strawberry, potato and tomato plants were crossed together and selected for plants containing in each case the fungus-resistant genes of both parents. In addition, transgenic plants were obtained by transforming them initially with one and then with one or more other gene. Finally, plants were also transformed with vectors which contained various resistance genes. Fungus-resistance tests were done with this plant material. Surprisingly, in all cases synergistic effects, not just additive effects, in respect of fungus resistance are observed.

For example, a tobacco plant which expresses ChiS and PSI shows a considerably greater resistance to Rhizoctonia infestation than the plants which expressed only ChiS or PSI or which would result from the additive resistance.

A synergistic inhibitory effect on infestation with *Rhizoctonia solani* also results from combined expression of PSI- and AFP-transgenic tobacco. Combination of two or more different genes (ChiS, RIP, AFP, ChiG and GluG) in a wide variety of transgenic plants also led to synergistic inhibitory effects on various fungi.

Whereas wild-type plants have index values from 38 to 46 in tests on 20 seedlings, it emerges with transgenic tobacco according to the invention that the latter grows as well in the presence of the fungus *Rhizoctonia solani* as do control plants (index value 10–12) cultivated on Rhizoctonia-free soil.